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MECHANISM OF CYTOTOXICITY OF THE AIDS VIRUS, HTLV-III/LAV

ANNUAL REPORT

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Understanding the mechanism of human immunodeficiency virus (HIV) type 1 replication and cytopathicity is critical to determining the pathogenesis and treatment of the acquired immunodeficiency syndrome (AIDS) and associated diseases. Studies of related viruses, simian immunodeficiency virus (SIV) and HIV-2, complement these studies and allow additional animal model systems for testing hypotheses. We have studies three aspects of HIV pathogenesis. First, a mutagenic analysis of HIV-1, SIV, and HIV-2 genes (including gag,env, vif, nef, and vpx) has been performed to decipher their role in replication and cytopathic activity. Second, immunopathogenetic consequences have been studied with respect to the charaterization of a lymphotoxin-like molecule which is secreted by HIV-1 infected cells. Third, the structure and function of HIV-1 env variation has been studied in a clustered outbreak of HIV-1 infection to elucidate the role of envelope alterations in immune evasion and tissue tropism.

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Foreword

1) Citations of commerical organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

2) For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

3) The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

Background and Significance

Human immunodeficiency virus (HIV) type 1 is a complex virus with at least three structural and five regulatory genes (1,2). The structural genes include gag, pol, and env. The 53 kilodalton (kd) gag precursor protein is encoded by the 9.0 kb full-length viral mRNA. It is cleaved by the viral protease to a 17 kd myristylated protein, a 24 kd phosphorylated major capsid protein, a 9 kd nucleic acid binding protein, and a 7 kd proline-rich protein. A 180 kd gag-pol precursor is synthesized from the same mRNA by ribosome frame-shifting occuring in the region of overlap between the gag and pol genes. The pol proteins processed from this polyprotein include a 10 kd aspartyl protease, a 55 kd reverse transcriptase, a 61 kd reverse transcriptase-RNase H, and a 32 kd endonuclease or integrase. The 160 kd envelope protein is encoded from a 4.5 kilobase (kb) spliced mRNA. The primary translation product is 863 amino acids long, including the first 30 amino acid signal peptide which is cleaved off. It is therefore estimated that this glycoprotein is 40% carbohydrate. The envelope precursor is processed by a cellular protease to a 120 kd extra-cellular envelope protein with 481 amino acids, and a 41 kd transmembrane protein with 345 amino acids.

At least five genes encode regulatory proteins. The transactivator protein, tat, gene is encoded by a double or triple spliced 2.0 kb mRNA. It is a 14 kd protein which is both highly basic and includes an array of cysteine and histidine residues characteristic of "zinc fingers." It interacts with the tat-responsive region, tar, localized between nucleotide 1 and 34 within the R region of the long terminal repeat sequences (LTR) and/or the mRNA (3). Tat increases gene expression about 1000-fold. However, its mechanism of action is not clearly defined, and may include effects on the initiation or elongation of transcription, RNA stability or processing effects, and/or effects on translation (4,5).

The regulator of virion protein production (REV) is an 18 kd protein encoded from a 2.0 kb mRNA. It acts as a strong positive-feedback regulator of virus expression increasing protein synthesis about 100-fold. Its mechanism of action is not understood.

The virion infectivity factor gene (vif) is likely encoded by a 5.0 kb mRNA. It is translated into a 23 kd protein. This protein acts at a post-translational step to markedly increase the infectivity of the virus particle (6).

The negative factor gene (nef) is encoded by a double or triple spliced 2.0 kb mRNA. It encodes the synthesis of a myristylated and phosphorylated 27 kd protein (7). This protein acts as a negative regulator of virus replication (8).

The mechanism of expression of the vpr gene product is not well understood (9). The function of this protein is not yet defined.

Lastly additional potential genes exist, including one designated vpu which may be encoded from the minus strand of the proviral DNA, and another which may be encoded from the plus strand of the proviral DNA (10).

The mechanism of cell killing by HIV-1 is likely a critical event in the development of immunosuppression in vivo. Indirect experimental data suggests an important role for both the HIV-1 envelope and the cellular T4 antigen. The extracellular envelope domain is critical for interactions with T4 and the formation of syncytia (11). The transmembrane domain serves to anchor the extracellular envelope on the infected cell or virion, as well to have a role in cell fusion, and a separate role in cell killing. The latter activity determined by the carboxyl terminal domain of the transmembrane envelope protein has been explored in these studies (12). The possible role of interactions of T4 and envelope independent of syncytia formation have also been studied here with respect to their role in cell killing. The development of new assays for cell killing independent of virus replication has been critical to these studies.

The role of the cellular gene products in cell killing also remains to be determined. Though viral product might cause direct lysis of infected cells through membrane effects, other mechanisms are more consistent with experimental data. Induction of cytopathic cellular proteins may explain the cytopathic effects of the virus and are likely to be more consistent with data obtained from both in vitro and in vivo studies (13). Several of these possible mechanisms have been explored in the studies outlined below.

Lastly the interaction of the virus and the immune system is critical to the pathogenesis of HIV-1 infections in vivo, and to the development of vaccines for this disease. It is clear that HIV-1's are a heterogeneous collection of related viruses, which differ more in the extracellular envelope product than other viral proteins (14). However, the functional signficance of envelope sequence variation with respect to rate of virus replication, infectivity, tissue tropism, responsiveness to neutralizing antibodies and other immune responses reamins to be determined.

Experimental Methods and Results

Analysis of HIV-1 Replication and Cytopathicity by Mutagenesis

Gag

We have been interested in the role of gag p17 myristylation. In the cases of Moloney murine leukemia virus and Mason Pfizer monkey virus, alteration of the myristylation acceptor site in gag leads to a marked decrease in virus replication, and loss of assembly and release of mature virus particles (15,16). It is likely that myristylation of gag p17 is critical to HIV-1 replication and that methods of inhibiting myristylation in vivo may provide anti-viral therapies. Thus, to test the role of gag myristylation in HIV-1 replication, we have constructed an M13 clone with this portion of the HIV-1 genome. It has been mutagenized by the method of Kunkel (17), to substitute the glycine codon at the second codon position of gag to an alanine. The resultant mutant fails to give rise to infectious virus particles. The mechanism of this effect was studied. We have demonstrated that myristylation is required for membrane targetting of the gag precursor and for subsequent proteolytic cleavage.

Env

We have begun three types of analyses of specific domains of the envelope gene. The first set of studies examine the role of the carboxyl terminal domain of gp41 in virus replication and cytopathicity. Two previously constructed mutants demonstrated virus replication in the absence of detectable cytopathic effects (12). The low percentage (1-2%) of syncytia formed in cultures infected by these viruses was similar to that found in cultures infected by the parental viruses. These clones were designated X10-1 which had a deletion of 5 amino acids of envelope replaced by 15 amino acids, and X9-3 which had a deletion of 5 amino acids of envelope replaced by 153 amino acids. A clone with a large deletion of env (about 50 amino acids), designated delta-E demonstrated greatly attenuated virus replication. The reason for the diminished cytopathicity of these viruses was not related to an alteration of interaction with T4 as demonstrated by the formation of syncytia using number of different lymphoid cell types. A defect in envelope protein synthesis or processing was also not detected as demonstrated by immunoprecipitation analysis of 35S-methionine labeled cultures infected with these virus strains. Thus, we felt that it is likely that the carboxyl terminus of gp41 has a separate enzymatic function separate from the ability of envelope to interact with T4 or form syncytia, and

that this activity was critical for cytopathicity. Though T4-envelope interactions were shown not to be sufficient for this cytopathic activity, it could not be ruled out that such interactions are necessary for the activity. We next asked whether envelope-T4 interactions may occur on the same cell, since these interactions may be critical to cytopathic effects. Thus, we asked whether capping either envelope or T4 using monoclonal antibodies and cross-linking them with a second antibody, led to internalization of the other protein. For this purpose, we have determined the concentration dependence of antibody binding to these proteins to obtain saturating levels. These concentrations of antibodies are in use in the co-modulation experiments at this time.

We also asked what portions of the carboxyl terminus of gp41 were important for virus replication and cytopathic effects. Thus, additional mutants have been constructed by Bal 31 nuclease treatment as previously described with or without the addition of a termination codon Xba I linker, their structure determined by nucleotide sequencing, and summarized in Table 1. Preliminary data with regards to the functional activity of these mutants performed in collaboration with Drs. Sue Jan Lee, Amanda Fisher, David Looney, and Flossie Wong-Staal (NCI) are summarized on the same table. Several interesting phenotypes are readily apparent. First, large deletions or additions (>10 amino acids) to gp41 lead to greatly attenuated virus replication in both Molt 3 and H9 cells. Several viruses, however, replicate well in H9 cells but not Molt 3 cells. Thus, differing modes of transmission, i.e. cell-to-cell versus free virus transmission, may be dependent on the conformation of gp41. All of the mutants with alterations of TM demonstrated reduced cytopathic activity.

We have also constructed mutants in the N-terminal domain of envelope. These utilized Bal 31 deletions from a unique Nde I site in a sor-minus clone, HXB2gpt-deltaS (6). The structure of each mutant was determined by nucleotide sequencing, and the data is summarized on Table 1. Notably, 3 clones have in-frame deletions in envelope. Clone MP79 has a deletion of amino acides 10-102, and thus a loss of most of the signal peptide. Clones MP22 and MP76 have slightly different deletions of the first conserved domain without affecting the signal peptide, including amino acids 36-105 and 35-107, respectively. Clone MP76 has been transfected into COS-1 cells and then cocultivated with Molt 3 cells. Virus replication was demonstrated by syncytia production and reverse transcriptase assays, though it was attenuated.

In additional studies of HIV-1 cytopathic effects, we have established a new assay system for measuring cytopathic effects in the absence of virus replication. For this purpose we have constructed a T4 expressing HOS cell line, and have been provided the T4 expressing HeLa cell line constructed by Maddon and colleagues. Transfection of HIV-1

demonstrated a 3-5-fold difference in virus production as measured by reverse transcriptase or p24 antigen assays in a single cycle of virus replication. Similar results were obtained with HeLa and SW480 colon carcinoma cells. F- and F+ virus derived from COS-1 cells showed similar infectivity on H9 cells as measured by the production of viral DNA within 12 hrs after infection. We have demonstrated that nef is a trans-activating factor. Cell co-transfected with an F- HIV-1 DNA clone and an F expression plasmid have the same phentoype as F+ transfected cells. Thus, these data demonstrate that the effects of nef in down-regulating virus production occur exclusively in the latter half of the virus replication cycle. Studies of gag p24 antigen production by immunoprecipitation studies of F- and F+ infected COS-1 cells again revealed a 3-5-fold difference, suggesting that F operated at a translational or pre-translational step. Studies of HIV-LTR-CAT assays in the presence or absence of tat with or without nef demonstrated downregulation of LTR directed gene expression. The effects of nef have been localized to the level of transcription by Northern blot analysis and nuclear run-off experiments.

A myristylation minus mutant of nef has also been obtained by site directed mutagenesis for analysis of the role of this post-translational modification in F activity. Furthermore, we have constructed 4 mutants in the presumptive GTP binding site of NEF, in the phosphorylation acceptor site at position 15, and 5 additional sites predicted to affect GTPase activity.

Vpx

HIV-2 and SIV differ from HIV-1 in that they contain an open reading frame encoding a 14 kd protein designated X (18). We have constructed functional clones of both HIV-2 and SIV, as demonstrated by the production of syncytia and reverse transcriptase in transfected H9 cells and in the case of HIV-2 in transfected CEM cells as well. Three mutations have been constructed in the vpx gene of HIV-2 (Appendix 5). These mutations do not affect the infectivity, replication rate, or cytopathicity of vpx in a wide range of lymphoid and monocytoid cell lines and primary human lymphocytes and monocytes.

Immunopathogenesis of HIV-1

We have performed two studies of HIV-1 lymphokine production. We have identified a lymphotoxin-like molecule secreted by HIV-1 infected peripheral blood mononuclear cells (13). Though some lymphoid cell lines constitutively excrete such a substance, no increase was detected after HIV-1 infection, despite the presence of cytopathic effects. Cell fractionation studies by panning of peripheral blood mononuclear cells failed to demonstrate in a convincing fashion which cell type was responsible for secretion of this agent.

It is known that lymphotoxin, tumor necrosis factor, and dexamethasone activate cell death via activation of a cellular nuclease and DNA degradation (19). We have examined whether HIV-1 produces a similar DNA degradation pattern. Though we succeeded in confirming the results of dexamethasone in induction of DNA degradation in human lymphoid cells, we failed to identify DNA degradation in HIV-1 infected cultures undergoing cytopathic effects. These data would suggest that the programmed mechanism of cell death involving cellular nuclease activation is not involved in HIV-1 induced cytopathic effects.

Interaction of HIV-1 and the Immune System

We have undertaken two types of studies of the interaction of HIV-1 and the immune system. First, we are establishing a murine model for study of cytotoxic responses to HIV-1. For this purpose we have established an AKR-2B murine cell line expressing the HIV-1 envelope. This was performed by transfecting an env expression clone with a selectable marker into these cells, and selecting a clone with expressed the HIV-1 envelope as detected by immunofluorescence studies with a specific antibody. These cells are being used as a target to study the cytotoxic responses of vaccinia-env vaccinated mice in collaboration with Dr. R. Markham (Washington University).

Second, we have studied a clustered outbreak of HIV-1 infection in 3 children who received HIV-1 contaminated blood via transfusion from an asymptomatic HIV-1 infected donor (Table 2). We have cultured virus from the blood donor (patient 1) and three of the recipients (patients 5, 6, and 7). All three patients have now developed AIDS and patients 5 and 6 have died of their disease. The virus isolates from each of these individuals is indicated in Table 3. Interestingly, the virus isolates from the lung and brain of patient 5 obtained in 9/86 grew well in monocytes, whereas those from the blood obtained in 2/86 and 8/86 from the same patient failed to grow at high levels in normal monocytes. DNA has been prepared from cultures infected with isolates from each of the patients, and libraries have been screened

for HIV-1 env clones from blood isolates from 2/86 and 8/86 of patient 5, lung isolate from patient 5, and blood isolate from 8/86 from patient 1. Three-ten clones have been obtained from each library and have been purified. Each clone has been excised in plasmid form for use in dideoxy sequencing. We have now sequenced 13 clones from 7 isolates from patients 1, 5, and 6 from isolates of brain, blood, and lung. We have detected only 3 amino acid changes in envelope, among the clones. No differences were identified in nef and LTR sequences among isolates from brain or blood. These data suggest that sequence heterogeneity is more restricted than that described in the literature. Second, these data suggest that monocyte-tropism is likely not to be based exclusively on sequences in env, nef, or LTR.

To further elucidate the basis for monocyte tropism, we have in collaboration with Dr. Howard Gendelman (WRAIR) obtained a 3' proviral clone from a monocyte tropic clone, designated ADA. The complete sequence of env, nef, and LTR has been determined. Genomic libraries have been constructed with DNA from the lymphocyte tropic virus counterpart of ADA.

To assess the biological basis of monocyte tropism, two studies have been initiated. First, we have cloned the LTR from the clone from the brain isolate of patient 5, and the LTR of the ADA monocyte-tropic isolate 5' to the CAT gene. We are currently establishing the most efficient method of transfection of primary monocytes to examine whether tissue tropism may be due to LTR activity. Second, we have constructed chimeric proviruses between HXB2 (lymphocyte-tropic) and either env-nef-LTR of clone of brain isolates of patient 5, env of clone of brain isolate of patient 5, or a full proviral clone from the brain isolate of patient 5. For this purpose, two termination codons in env are being corrected to trp by site directed mutagenesis. Chimeric proviral clones have also been constructed with HXB2 using the env-nef-LTR sequence of ADA monocyte tropic clone.

Conclusions

Work performed during the last year under this contract has better defined viral determinants, cellular determinants, and interactions of viral and cellular determinants in HIV-1 induced cytopathicity.

In studies of viral determinants of HIV-1 replication and cytopathicity, we have produced a number of mutants to better characterize the function of particular gene products. A myristylation acceptor mutant of gag has demonstrated the requirement of myristlation for membrane targetting of the gag precursor, proteolytic cleavage, and infectious virus production. Similar results have now been obtained with sulfur and oxygen substituted analogs of myristic acid.

We have constructed a number of mutants with an altered carboxyl terminus of gp41. These have demonstrated that additions or deletions of more than 15 amino acids attenuates infectivity and/or replication. Replication of certain mutants occurred in H9 cells but not Molt 3 cells suggesting that a region within the carboxyl terminus of gp41 may be critical to cell-to-cell transmission of virus but not transmission via free virus particles. Several of these mutants should be useful for characterizing regions in the carboxyl terminus of gp41 which affect cytopathicity without affecting virus replication. A number of mutations in the first conserved domain of gp120 have also been produced. Two of these with an in-frame deletion, gives rise to virus which replicates to attenuated levels. These mutants will better define the role of this portion of gp120 in infectivity, virus replication, and responsiveness to neutralizing antibodies. Interactions of envelope with T4 may be critical for cytopathicity. We have determined experimental conditions which are appropriate to study whether co-modulation of T4 and env occur with cross-linking antibodies which would suggest an interaction on the same cell. We have also established an assay for cytopathicity independent of virus replication using sub-genomic proviral clones transfected into HeLa or HOS cells expressing T4. This assay should be useful in defining viral determinants of cell killing and their mechanism of action.

Studies of vif have demonstrated that it acts at a post-translational level to increase the infectivity of the virus particle. A number of vpr mutants have also been constructed. Our data suggests that they all give rise to virus which replicates and kills lymphoid cells similar to the parental virus. However, in MT4 cells, vpr is contributes to the infectivity of the virus.

Studies of nef have demonstrated that it is negative a regulator of virus replication acting at the level of viral RNA synthesis. Additional studies should better characterize its mechanism of action. Site directed mutants in the myristylation acceptor codon, phosphorylation acceptor codon, and presumptive GTP binding and GTPase domains of nef have been constructed to assess the role of these activities in regulation of HIV-1 replication by nef.

HIV-2 and SIV have an additional gene, designated vpx. We have constructed several HIV-2 and SIV functional clones to test the effect of mutation of the vpx gene. We have constructed a three HIV-2 X mutants. No alterations in infectivity, replication, or cytopathicity has been detected.

In studies of cellular determinants of HIV-1 cytopathicity, we have identified a lymphotoxin-like factor secreted by HIV-1 infected cells. Its role in cytopathicity is unclear at this point. We have failed to detect DNA fragmentation in HIV-1 infected cultures, similar to that which occurs in glucocorticoid or lymphotoxin treated cells.

To examine interactions of viral and cellular determinants of cytopathicity, we have focused on the significance of HIV-1 envelope sequence heterogeneity. We have isolated virus from 3 children who were infected from a single blood donor. Virus was isolated at different times and from different tissues. Interestingly, virus isolates from the lung and brain of one child was monocyte-tropic whereas that isolated from the blood grew poorly in monocytes. Env clones have been obtained from lung and blood isolates from one child, lung, brain, and blood isolates of a second child, and from two blood isolates of the donor. Sequencing of 13 clones from these seven isolates has demonstrates <0.02% amino acid variation in envelope. The validity of these data have been corfirmed by sequence analysis of sequences obtained by PCR directly from fresh tissue. Furthermore, no differences in env, nef, or LTR are apparent to explain monocyte tropism of certain isolates. A clone has also been obtained from the monocyte tropic ADA isolate and sequenced. LTR CAT clones have been constructed as well as chimeric proviral clones between clones from lymphocyte-tropic and monocyte-tropic clones.

Recommendations

We recommend the following studies be carried out over the next year.

1) With respect to the studies of the role of gag in virus assembly, we will carry out several studies over the next year:

a) We will examine the role of myristylation in targetting the gag precursor to membranes in further detail. In our initial experiments, w fractionated cells between membranes and cytosol, and have detected an alteration in the proportion of gag precursor in the membrane fraction in the absence of myristylation. We will further fractionate this membrane fraction into plasma membrane and intracellular membrane fractions to further delineate the role of myristylation. We will also carry out studies by immunogold electron microscopy and by immunofluorescence to further define the role of myristylation in the targetting of the gag precursor.

b) We will assess the role of envelope in targetting gag to the membrane. The initial studies were carried out in the presence of envelope protein synthesis. We will repeat the studies in the absence of envelope protein synthesis to determine if the envelope influences targetting of gag to the membrane. If so, we will then examine mutants of envelope, and particular TM, and mutants in gag to assess the role of each determinant in this targetting activity.

c) We will assess the role of binding of virion RNA to gag in targetting gag to the membrane. For this purpose, we will attempt to inhibit virion RNA binding by deleting a presumptive packaging sequence in the 5' leader sequence or by deleting codons in the nucleic acid binding protein that are thought to contribute to packaging.

d) We will assess the role of phosphorylation of gag p17 and p24 in gag targetting and assembly. We will individually mutagenize Ser residues through these coding domains to identify those which are phosphorylated and to elucidate their role in virus assembly.

2) With respect to the studies of the HIV-1 envelope, we propose to perform several experiments:

a) We will analyze the cytopathic activities of each of the carboxyl terminal gp41 mutants, their susceptibility to neutralizing antibodies, the interaction of their envelope with T4, and their cytopathic activities.

b) We will utilize saturating concentrations of antibodies to envelope or T4 in the co-modulation experiments to look for interactions of envelope and T4 on the same cell type. If this is identified, we will examine co-modulation with each of the carboxyl and amino terminal mutants of envelope that we have constructed.

c) We will replace the deleted vif gene in each of the clones with deletions in the first conserved domain of the N-terminus of envelope and examine the abilities of the clones derived from MP22 and MP76 to give rise to virus, its replication rate, and cytopathic activities. We will also transfect each of the clones already available and listed in Table 2 into COS-1 cells either alone or together with an env expression clone to assess the role of the first conserved domain and all of envelope in virus production.

d) We will assess the cytopathic activities of clones derived from HIV-1 by measuring the number of gpt+ clones obtained after transfection of HeLa/T4 and/or HOS/T4 cell lines. These assays will seek to identify cytopathic genes in HIV-1 independent of virus replication.

e) We will look for interactions of gp41 with other viral and cellular proteins to try to identify mediators of the cytopathic activity. This will be performed by cross-linking experiments using 35S-methionine labeled cells and specific antibodies to gp41 which have been either purchased commercially (DuPont, Epitope) or provided by other investigators (Dr. Kennedy, Southwest Medical Foundation).

f) We will construct mutants in several interesting domains of gp41 (TM) to elucidate their role in cell-cell fusion and virus infectivity. These domains will include the three cysteine residues, the three potential N-glycosylation sites, the amphipathic helices in the carboxyl terminal portion of the molecule, and the hydrophobic domain at the N-terminus.

3) In our studies of vpr, we will carry out several experiments:

a) We will determine the effect of vpr on infectivity in MT4 cells. We will assess whether the mutation may be complemented in trans by a vpr expression clone. We will determine if the vpr activity is through an interaction in vif by examining whether vif mutants can complement vpr mutants. We will examine binding of mutants of vpr to MT4 cells.

b) We will assess whether vpr affects infectivity in other cell lines, including other HTLV-I infected cell lines, e.g. MT2, HUT102 cells, as well as MOLT 4 cells. Studies will also be carried out in primary lymphocytes and when a monocyte tropic HIV-1 proviral clone is available, in primary monocytes.

c) We will attempt to detect the vpr protein in infected cells by immunoblot with an antibody to a recombinant protein product of vpr or with antisera from patients. We will also make polyclonal antibodies to synthetic peptides and other bacterial expression products of vpr. After identification of the vpr protein, we will examine its cellular localization, and determine whether it is present in the virion.

d) We will examine the role of vpr with in vivo model systems. For this purpose, we have constructed vpr mutants in HIV-2 and will construct similar mutants in functional proviral clones of SIV to allow testing in rhesus macaques. Furthermore, we will examine the role of vpr in HIV-1 replication in scid/hu mice.

4) In our studies of nef, we will perform the following experiments:

a) We will determine the sequences in the HIV-1 LTR which are responsive to NEF utilizing CAT constructs with HIV-1 LTR deletion mutants. We will also utilize gel retardation, footprinting analyses, and in vitro transcription. The source of nuclear proteins for these studies will be HeLa and Jurkat cell line which have been transfected with nef expression clones.

b) We will determine the functional sites of F by analysis of a variety of site directed mutants with regards to negative regulation of virus replication or the particular step in virus replication that is involved and GTP binding activity in transfected COS-1 cells.

c) We will also perform in vivo phosphorylation experiments with F- and other mutants to assess the role of F in phosphorylation of viral and cellular proteins. We will particularly focus on phosphorylation of gag p24 and F itself.

d) We will assess the myristylation acceptor minus mutant of HIV-1 in the same way already described for the other F minus mutants. Cell fractionation experiments are likely to be utilized to define whether myristylation regulates via an intracellular sorting mechanism or by other means.

e) We will construct a retrovirus expression vector which synthesizes high levels of NEF to assess its role in down-regulation of HIV-1 replication.

5) In our studies of the SIV and HIV-2 vpx genes, we will carry out the following studies:

a) We will complete the construction of SIV vpx mutants and assess their influence on infectivity, replication, and cytopathicity in vitro.

b) We will assess the role of vpx mutants of SIV and HIV-2 in virus replication in rhesus macaques and scid/hu mice.

6) In the studies of a murine model of cell mediated immunity, we will collaborate with Dr. Richard Markham (Washington University) to assess cytotoxic T cell responses to AKR cells expressing the HIV-1 envelope. A similar target system will be developed utilizing murine Lyt3+ cells expressing the HIV-1 envelope.

7) In the studies of envelope variation and its significance in the clustered HIV-1 outbreak, we will carry out several studies:

a) We will clone the lymphocyte tropic variant of ADA as well as obtain additional clones of the monocyte tropic variant. The env, nef, and LTR sequences will be determined. These clones will be utilized for functional studies as already described.

b) We will transfect the LTR-CAT clones into primary monocytes or primary lymphocytes to assess the contribution of these sequences to monocyte tropism.

c) We will transfect the chimeric proviral clones into COS-1 cells and use the resultant virus to infect monocytes or lymphocytes. We expect that these experiments will identify a monocyte tropic clone, and sequences within that clone that confer monocyte tropism to a lymphocyte tropic clone. Further studies on the mechanism of action will depend on which sequences are identified to be important for monocyte tropism.

8) We have initiated studies to define the packaging sequence for virion RNA, as described above. If successful, a packaging cell line will be constructed, and viral particles from this cell line will be utilized for vaccine studies in chimpanzees. The cell line will also be transfected with a proviral clone in which the hygromycin gene has replaced a portion of env. The resultant virus should carry a genome with a hygromycin gene. This virus will then be used to infect murine T4 cells which have been transfected with HeLa cell DNA. Hygromycin selection will allow selection of cells that allow uptake of virus, and these cells will be used to identify and characterize the human sequences responsible for this activity.

9) In collaboration with Richard Markham (Johns Hopkins) and Michael McCune (Stanford), we have initiated experiments to examine each of the viral variants described above in scid/hu mice.

N-TERMINAL ENVELOPE DELETION CLONES

Clone	Frame-shift	Nucleotides Deleted	No. Nucleotides Deleted	Amino Acids Deleted	No. Amino Acids Deleted	Signal Peptide Intact	Mature Envelope Product
MP22	-	5907-6116	210	36-105	70	+	TEKLW/EDIISL...
MP76	-	5904-6122	219	35-107	73	+	TEKL/IISLW...
MP79	-	5829-6107	279	10-102	93	-	
MP7	+	5853-6133	281				
MP10	+	5867-6134	268				
MP19	+	5885-6125	241				
MP70	+	5912-6086	175				
MP82	+	5910-6136	227				
MP87	+	5833-6116	274				
MP92	+	5910-6125	216				
MP100	+	5832-6114	283				
MP122	+	5811-6118	308				
MP18	?	? -6131					
MP51	foreign sequence						

Clones to sequence MP74
Repeat sequence on MP18

Table 2

QUENCE HETEROGENEITY OF ISOLATES FROM A CLUSTERED HIV-1 OUTBREAK

Transfusion event:

One unit of blood from patient 1 infused in patients 2-7 in 2/85

Patient descriptions:

Patient 1 - Asymptomatic 21 year old white male homosexual
8/86 - T4 500 cells/cu mm

Patient 2 - Died in neonatal period of complications of prematurity,
HIV-1 assays not performed

Patient 3 - Died in neonatal period of complications of prematurity
HIV-1 assays not performed

Patient 4 - Died at age 5 months of respiratory syncytial virus infection,
HIV-1 assays not performed

Patient 5 - 30 wk gestation, intracranial hemorrhage, hydrocephalus
Age 8 mos - recurrent diarrhea, failure to thrive, generalized lymphadenopathy, hepatosplenomegaly, diffuse pulmonary infiltrate
Age 12 mos - panhypergammaglobulinemia, HIV encephalopathy with CT scan showing cerebral atrophy and basal ganglia calcifications
Age 18 mos - recurrent fevers, aspergillus otitis externa
Died in 9/86 at age 20 months

Patient 6 - 29 wk gestation, respiratory distress syndrome, brochopulmonary dysplasia
Age 5 mos - recurrent diarrhea, posterior cervical lymphadenopathy
Age 7 mos - failure to thrive, diffuse pulmonary infiltrates, generalized lymphadenopathy
Age 9 mos - pneumococcal meningitis
Age 16 mos - recurrent scabies
Died at age 30 mos

Patient 7 - 26 wk gestation, respiratory distress syndrome, bronchopulmonary dysplasia, patent ductus arteriosus
Age 5 mos - failure to thrive, generalized adenopathy
Age 11 mos - respiratory syncytial virus infection

Table 3

OLATES FROM A CLUSTERED HIV-1 OUTBREAK

Patient	Date	Tissue Source	Result of Culture
1	4/86	Blood	-
	7/86	Blood	-
	8/86	Blood	+
	9/86	Blood	-
	11/86	Blood	-
	1/87	Blood	-
	3/87	Blood	-
	6/87	Blood	-
5	2/86	Blood	+
	8/86	Blood	+
		CSF	nt
	9/86	Lung	+
		Brain	+
		Lymph node	-
		Spleen	-
6	2/86	Blood	+
	9/86	CSF	nt
	11/86	Blood	+
	2/87	CSF	-
	4/87	Blood	+
	6/87	Blood	+
		CSF	-
7	2/86	Blood	nt
	7/86	Blood	-
	11/86	Blood	+
	4/87	Blood	-
	11/87	Blood	-
	2/88	Blood	-
	3/88	Blood	-

eviations: nt, not tested; CSF, cerebrospinal fluid

ures were performed by mixing an equal number of patient peripheral blood nuclear cells weekly with normal phytohemagglutin-stimulating normal peri- al blood mononuclear cells. Positive cultures are considered positive if or more indepent culture supernatants are found to give reverse transcrip- values double the background. Positive cultures are confirmed by demon- ion of a 3.5 kb Sac I DNA fragment in cellular DNA which hybridizes with

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